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Organophosphorous pesticide exposures and sperm quality

Melissa J. Perry^a, Scott A. Venners^b, Xing Chen^a, Xue Liu^c, Genfu Tang^c, Houxun Xing^c, Dana Boyd Barr^d, and Xiping Xu^e

^aDepartment of Environmental Health, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115, USA

^bFaculty of Health Sciences, Simon Fraser University, 8888 University Drive, Burnaby, BC V5A 1S6, Canada

^cInstitute for Biomedicine, Anhui Medical University, 81 Meishan Road, Hefei 230032, China

^dDepartment of Environmental and Occupational Health, Rollins School of Public Health, Emory University, 1518 Clifton Road, NE, Room 272, Atlanta, GA 30322, USA

^eSchool of Public Health, University of Illinois at Chicago, 966 SPHPI, M/C 923, 1603 W. Taylor St, Chicago, IL 60612, USA

Abstract

Many Americans are exposed to low levels of organophosphorous (OP) pesticides. It is unclear whether these exposures impact sperm production. We investigated whether there was an association between urinary OP insecticide metabolites and sperm concentration and motility in newly married men from a rural area of eastern People's Republic of China. Ninety-four cases and 95 controls were included based on their median residual value of sperm concentration and motility after adjusting for relevant covariates. Their urine was analyzed for six dialkylphosphate (DAP) compounds. After adjustment for demographic and exposure variables, the odds of being a case were greater (Odds Ratio=1.30, 95% Confidence Interval 1.02-1.65) in men with higher urinary concentrations of dimethylphosphate (DMP) compared to men with lower levels. No significant differences between cases and controls were found among the other DAP concentrations. DMP exposure and sperm concentration and motility should be explored further in environmental exposure studies.

Keywords

Organophosphorous; Insecticides; Pesticides; Reproduction; Semen quality; Hormones; Chinese; Male

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Corresponding author: Melissa Perry, ScD, MHS, Department of Environmental Health, Harvard School of Public Health, 665 Huntington Avenue, Building 1, Room 1411, Boston, MA 02115, Telephone: (617) 432-4645, Fax: (617) 432-0219, mperry@hsph.harvard.edu.

Author agreement

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The authors of this article confirm they do not have any conflicting financial or nonfinancial interests in its content.

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1. Introduction

About 73 million pounds of organophosphorous (OP) pesticides were used in the United States in 2001 (70% of all insecticides) [1] and approximately 35 organophosphate insecticides are currently registered for use in the United States by the U.S. Environmental Protection Agency. While global quantities are not well documented, OP pesticides are used widely in agriculture and in private homes for controlling household insects, particularly in Latin America, Africa, and Asia.

Chemically, OP pesticides are esters of phosphoric or thiophosphoric acids and can be toxic to mammals because of their capacity to phosphorylate acetylcholinesterase, causing accumulation of acetylcholine in synapses. Some OP pesticides have been found to have endocrine disrupting properties [2]. Epidemiologic studies have suggested associations between OP exposure and reproductive disorders (infertility, birth defects, adverse pregnancy outcomes, perinatal mortality)[3]. OP pesticides are suspected to alter reproductive function by reducing brain acetylcholinesterase (AChE) activity and secondarily influencing the gonads. They have been shown to alter pituitary–thyroid and pituitary–adrenal axes and affect prolactin serum levels [4,5]. OP pesticides such as parathion and methyl parathion are structurally similar to various hormones, including estrogens and may interact with hormone receptors and/or affect gene transcription. These compounds bind to the oestrogen receptor protein [6], and this is one hypothesized mechanism by which they exert oestrogen agonist effects.

To date few studies have evaluated the impacts of OP pesticide exposure specifically on male reproductive function and most have studied pesticide sprayers [7-11]. For example, prior epidemiologic work from our group showed that in Chinese pesticide factory workers, OP pesticide exposure was associated with decreased sperm concentration and motility [12], increased luteinizing hormone and decreased testosterone [13], and higher sex chromosome aneuploidy in sperm [14].

We conducted a cohort study of recently married couples in rural China to study male and female reproductive health and pregnancy outcomes. Our initial biomonitoring results showed that OP pesticide exposure was common among the men in our cohort and that exposures were predominately through environmental routes [15]. The objective of this nested case control study was therefore to evaluate the association between OP pesticide exposure, as assessed by dialkylphosphate (DAP) urinary concentrations, and semen parameters in men environmentally exposed to pesticides in rural China.

2. Methods

We used case control methodology to study male partners in the cohort study. Cases were identified as men having semen concentration and sperm motility values below the median population values after adjusting for potentially important covariates and controls were identified as men with semen and sperm motility values above the median population values. The following sections detail how the men were recruited, how cases and controls were selected, and how the data were analyzed.

2.1 Study participant recruitment

We recruited subjects from July 2003 to February 2005 from agricultural regions in Anhui Province, which were selected due to their close proximity to Anqing City, the location of the Anhui Medical University Institute of Biomedicine. The study protocol was approved by the Harvard School of Public Health Human Subjects Committee. All recruitment and data

collection procedures were carried out by trained medical personnel from the Anhui Medical University. After obtaining contact information from registrations of marriages with the provincial government and planned pregnancies with the family planning bureau, we contacted couples at their homes. Additionally, we approached couples who were attending clinics for prenatal care. After obtaining oral consent, we explained the study and invited couples to participate. Those who agreed to participate were invited to a field office at a later date for baseline procedures, which commenced only after the study was explained again in detail, couples had an opportunity to receive answers to any questions they had about the study, and both signed a written consent.

For all couples enrolled in the study, the inclusion criteria were: 1) the marriage was the first for both the wife and husband; 2) the wife's age was between 20 and 34 years; 3) the wife was not a smoker and had never been one in the past; 4) both the wife and husband were available for the study, and 5) the couple currently lived together or planned to live together after marriage. Couples were eligible for inclusion in the study if they planned to stop contraception (or begin sexual activity) and try to conceive in the near future. We also enrolled pregnant couples if no more than two months had passed since the wife's previous menstrual period. All subjects who met the inclusion criteria were invited to participate in the study.

As is now common throughout China, most young couples from this agricultural region had migrated to urban centres for employment. However, according to cultural custom, most returned to their native homes during the period around the lunar New Year and it was common for many young couples to register their marriages and marry during this period. Therefore, 75% of our recruitment occurred within the 60 days prior to the lunar new years which were January 22, 2004 and February 9, 2005.

2.2 Study procedures

On the day that the couple visited the field office, each was given a routine physical examination and blood and urine samples were collected. Both answered questions from a trained interviewer using a structured questionnaire including socio-demographic characteristics, history of diseases, general health status, active and passive cigarette smoking, alcohol consumption, tea drinking, and chemical exposures at work.

Semen was collected in a sterile container by masturbation in a private room near the analysis laboratory. Husbands were asked to abstain from ejaculation for three days prior to the scheduled date of collection and the importance of accurately reporting the time and date of the previous ejaculation was stressed. The time of abstinence was calculated to the nearest minute using the time and date of semen collection minus those of the previous ejaculation as reported by the husbands. All semen analyses were supervised by a laboratory technician who had been previously been trained by US certified andrologists. Sperm volume was measured in a graduated cylinder with a canonical base. Sperm counts were determined on two separate drops of semen using a Neubauer hemacytometer after dilution according to World Health Organization protocols [16]. If the sperm counts determined in the two drops of semen differed by 10% or more, then the count was determined in a third drop of semen. In this case, the sperm count from the first two samples which was closest and within 10% of the third count was retained. Sperm count was calculated as the average of the two sperm counts. Sperm motility was determined microscopically in two 10 µl drops from the same semen sample according to WHO protocols. In each sample, 200 spermatozoa were graded for total motility, which included grades a, b and c according to the WHO protocol: (a) rapid progressive motility, (b) slow or sluggish progressive motility, (c) non-progressive motility, but excluding (d) immotility. If the percentage total motility (a+b+c) differed in the first and second drop by more than 10%, then motility was assessed in a third drop. Sperm

morphology slides were prepared and stored but analyses are incomplete. The validity of the procedures was verified by analysis of duplicate samples from a certified andrology laboratory in the United States.

2.3 Case and control selection

Cases and controls for this analysis were selected prior to the completion of study participant recruitment at which time there were 604 males in our database who had provided a semen sample. We excluded 259 men from possible selection as cases or controls because they did not have sperm motility data ($n=202$ because sample did not liquefy at room temperature); repeated measures of concentration or motility differed by more than 15% ($n=29$); they had no motile sperm ($n=6$); or they had a history of pelvic inflammation, blood disease, or malignant tumour ($n=5$). We have yet to determine why such a sizable number of samples failed to liquefy but are investigating reproductive tract infections as a possible cause. We also excluded 11 men because their samples were not collected in the winter (to limit seasonal variation in sperm characteristics) and 6 because they were of Hui ethnicity (whereas all other men were of Han ethnicity). We selected cases and controls from the remaining 345 men and these men did not statistically differ from the 257 excluded men in age, education, smoking, alcohol use, or self-reported exposure to chemicals at work.

Our goal was to select men as controls if their sperm concentration and motility were high after adjusting for important factors that could affect sperm and conversely, to select men as cases if their sperm concentration and motility were low after adjustment. We first used linear regression to regress log sperm concentration (which was normally distributed) onto age, alcohol use, passive and active smoking amount, delay of sperm analysis after ejaculation, education, self-reported exposure to chemicals at work, and time passed since last ejaculation (linear and squared terms). It was important to regress on time passed since last ejaculation because 59% of the men had ejaculated within 2 days previously contrary to study protocol. We kept covariates that were significant (at $\alpha=0.05$) in our final model, which included active smoking and time since last ejaculation. We then ranked these men by their residuals in this model and compared men who were above and below the median residual value. The men differed in sperm concentration ($p<0.0001$ by t-test), but were not statistically different in passive and active smoking, alcohol use, or self-reported exposure to chemicals at work.

We next followed a similar procedure regressing sperm total motility onto the same covariates. Our final model included only delay of sperm analysis after ejaculation. We then ranked these men by their residuals in this model and compared men who were above and below the median residual value. Men below the median tended to have lower sperm motility (Mean $\pm$ SD = $54 \pm 14\%$) than those above ($80 \pm 6\%$), but this did not reach statistical significance at $\alpha=0.05$ ($p=0.07$). These groups did not statistically differ in age, time since last ejaculation, education, active and passive smoking, alcohol use, or self-reported exposure to chemicals at work.

For this analysis, 94 men were below the median residual values in both models (concentration and motility) and classified as cases and 95 men were above the median residual values in both models and classified as controls. Cases and controls differed in both percentage of motile sperm (53 ± 15 versus $79 \pm 6\%$, $p<0.0001$) and sperm concentration (17 ± 12 versus 79 ± 70 million sperm/ml semen, $p<0.0001$), but were similar in age, time since last ejaculation, education, active and passive smoking, alcohol use, time from ejaculation to semen analysis, and self-reported exposure to chemicals at work.

2.4 Biomonitoring of OP pesticides

Urine samples were analyzed at the Centres for Disease Control and Prevention, and analysts were blind to study objectives and to the individual characteristics of the study samples. Dialkylphosphate metabolites of OP pesticides were measured using a mass spectrometry-based method and quantification using the isotope dilution (ID) calibration [17]. This is the same method and the same national laboratory that monitors non persistent pesticides in the US general population, published in the Third National Report on Human Exposure to Environmental Chemicals [18]. Briefly, using ID calibration, the samples are enriched with isotopically labelled analogues prior to preparation. Chemically the isotope analogue behaves identically to the native analyte, but can be discriminated with a mass filter [19]. This allows complete recovery correction for each sample and improves the sensitivity, accuracy, and selectivity of the analysis. After addition of the labelled standard to urine samples, the urine samples are lyophilized and the DAP metabolites are derivatized to form their chloropropyl phosphate esters. The DAP esters are extracted from the residue using an immobilized liquid-liquid extraction. The extracts are concentrated to dryness and reconstituted in solvent for analysis by ID-gas chromatography-tandem mass spectrometric (MS/MS).

As indicators of OP exposure, six dialkylphosphate (DAP) metabolites: (DEDTP = diethyldithiophosphate; DMP = dimethylphosphate; DMTP = dimethyldithiophosphate; DEP = diethylphosphate; DETP = diethylthiophosphate; DMDTP = dimethyldithiophosphate) as described in Bravo et al. [17] were screened. Approximately 75% of organophosphorous compounds yield these DAP metabolites in urine. The limit of detection (LOD) for all analytes were determined and values too low to be quantified were assigned a value equivalent to the $LOD \times (2)^{-1/2}$. Results are reported utilizing creatinine adjustment.

2.5 Data analysis

Differences between cases and controls on demographic characteristics were compared using t-tests for continuous variables and chi-square tests for categorical variables. Descriptive statistics for metabolite concentrations by cases and controls included the percent above the limit of detection, mean and standard deviation, geometric mean and standard deviation, ranges, and calculation of the 25th, 75th and 90th percentile. All OP metabolite concentrations were log transformed.

We used PROC LOGISTIC in SAS to estimate the relative odds of being a case per 1 ug/L increase in OP metabolite concentration both with and without adjustment for the following covariates: age (linear), current alcohol drinking (drink/non-drinker), education level (<high school/high school/>high school), average daily cigarettes smoked (none/1-9/10-19/>=20 cigarettes), average daily cigarettes smoked by others around subject (passive smoking) (none/1-9/10-19/>=20 cigarettes), self-reported exposure to chemicals at work (yes/no), days of sexual abstinence prior to semen collection (linear and squared) and time delay from ejaculation to laboratory analysis of semen (<30/>=30 minutes). Results from the logistic models are reported as odds ratios and 95% confidence intervals.

The relationships between OP metabolite concentrations and sperm quality were visualized using a generalized additive model to show the odds of being a case with increasing OP metabolite concentrations. All of the smoothing plots used penalized splines from the GAM function of MGCV package in R as a nonparametric method. The binomial distribution, penalized thin plate regression splines were used in the GAM model, along with automatically selected degrees of freedom based on UBRE score.

3. Results

Table 1 details the demographic characteristics of the cases and controls and shows there were no significant differences between the two groups except for the case criteria of sperm concentration and motility.

Table 2 shows the frequency of detection of OP metabolites in the total sample. Ninety eight percent of controls and 94% of cases had detectable concentrations of DETP and 94% of controls and cases had detectable concentrations of DMTP. These urinary metabolite concentrations can result from exposure to a range of OP pesticides or their environmental degradates including azinophos methyl, chlorpyrifos, chlorpyrifos methyl, malathion, and methyl parathion [20]. 2004 pesticide sales data collected in our study area showed that methyl parathion, which is used for killing cotton-related pests, was one of the leading products sold.

Ten percent of the men in our sample reported applying pesticides in the past year, so we compared their metabolite concentrations using two sample t-tests to the rest of the sample and did not find significant differences.

Table 3 details the results of the case control analysis, showing that after adjustment for demographic and exposure variables, men with lower semen quality (cases) had significantly higher levels of urinary DMP as compared to men with higher semen quality (controls) (OR=1.30, CI 1.02-1.65). There were no other significant differences among the other metabolite concentrations between cases and controls.

Figure 1 illustrates smoothing plots using penalized splines, showing that DMP concentration had a linear pattern across case/control groups, confirming the odds of having low sperm concentration and low sperm motility increased with increasing DMP concentration.

4. Discussion

The biomonitoring results from this study indicate the majority of men had been exposed to OP insecticides or degradates that metabolize to DETP, DMTP, and DMP. Most of the DAP metabolites were not significantly associated with sperm concentration and sperm motility, with most odds ratio estimates less than 1.0 and 95% confidence intervals overlapping 1.0. Of note was a marginally significant association between DMP and semen quality (Odds Ratio=1.30, 95% Confidence Interval 1.02-1.65). This association was detected even though the prevalence of detections was comparable among cases and controls; however, metabolite concentration levels were higher among cases.

Previous epidemiologic studies of OP exposures and semen quality have mainly focused on occupational exposures among pesticide sprayers [7-10,12,22,23]. Four studies measured DAPs, and one of these studies among Peruvian pesticide sprayers [11] reported a significant association among semen volume, motility and morphology with DMPDMP levels were considerably higher among the Peruvian sprayers (median = 6.83 µg/G creatinine ~ 10ug/L) [8] than the median DMP concentration in our sample among men who were not pesticide sprayers (1.8 ug/L). Therefore this is the first study to report an association between environmental exposure to DMP and semen parameters, at levels lower than seen in previous occupational studies. US comparisons show the DMP levels seen in this study were higher at the median but comparable at the 90th percentile suggesting that at least 10% of the US population is being exposed at levels comparable to what was observed at the highest level in this rural China sample.

In terms of mechanisms of action, toxicologic studies have suggested that broadly, OPs can impact sperm concentration through damage to the seminiferous epithelium by affecting germ cell proliferation [24], and can impact sperm motility by disturbing the assembly of tail structural protein components and/or ATP synthesis [25]. Our review of the literature of studies specific to DMP and reproductive effects found one human study linking DMP to decreases in gestational age [26] and one rat study showing DMP was associated with decreased sperm motility but not morphology or concentration [27]. Given little prior information, understanding the connection between urinary DMP concentration and decreased semen quality warrants further study.

Limitations of this study are common to observational studies of environmental pesticide exposures. We had little information on the circumstances of OP pesticide exposure in our sample. These metabolites are not unique to any one parent OP compound and each of the six urinary DAP metabolites can be produced from the metabolism of more than one OP pesticide. For example, DMP can be metabolized from at least 17 different OP compounds [18]. Without other environmental exposure information, the presence of DAP metabolites in urine cannot be used to identify the exact source of OP pesticide exposure. In addition, urinary DAPs can be derived from exposure to the pre-formed metabolite in the environment. Also, it was not possible to determine when exposures took place in relation to the spermatogenesis cycle which typically last 60 days in the testis, with a further 10 to 11 days being required for passage of spermatozoa through the epididymis and vas deferens into the ejaculate [28]. Although critical windows of chemical insult in the human spermatogenic cycle are not well known, it is also unclear where men were located in the 60 days prior to sample collection when they were exposed to OPs. At the time of study recruitment most had recently returned to the agricultural region from other cities to spend the Chinese New Year holidays with their families. Additionally, using a one time semen sample to evaluate motility and morphology gives a limited characterization of how an environmental exposure may impact testis function. We were unable to collect repeat semen samples because the men in our study returned to their cities after the New Year holiday. The semen parameters from this study should not be used to compare overall semen quality in this population to other populations due to the high number of men who did not abstain for two days. Although semen parameters were lower in this study because of the shorter abstinence time as compared to other semen analysis studies, we did not find evidence that abstinence time varied by pesticide exposure and we also adjusted for abstinence time in our models. Therefore, our estimates of the effects of pesticide exposures on semen parameters were not confounded by abstinence time. An additional caution is that we tested multiple metabolites for their associations with sperm quality and p values were not adjusted for multiple tests so our results do not have global statistical significance. It will be important to investigate these metabolites further in future male reproductive health studies.

However, this is the largest study conducted to date to examine largely environmental OP pesticide exposures in the form of urinary OP metabolite concentrations in relationship to semen parameters. Unique features of this study included that the sample was population-based rather than clinic-based and the pesticide exposures were largely environmental rather than occupational. The cases and controls were selected from a very large sample of population based semen parameters, allowing representation of the extreme ends of a population-based distribution.

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Abbreviations

DEDTP	diethyldithiophosphate
DMP	dimethylphosphate
DMTP	dimethyldithiophosphate
DEP	diethylphosphate
DETP	diethylthiophosphate
DMDTP	dimethyldithiophosphate
OP	organophosphorous pesticides
OR	odds ratio

Figure 1. Spline of adjusted log odds of case status^a by log(DMP^b concentration) using a generalized additive model

^aControls were defined as individuals with both sperm concentration and total motility above the population medians after adjustment. Cases were defined as individuals with both sperm concentration and total motility below the population medians after adjustment.

^bDMP=dimethylphosphate.

Note: Adjusted for age, alcohol drinking, passive smoking, education, occupational chemical exposures, active smoking, abstinence from sexual activity, and time delay from ejaculation to laboratory analysis of semen.

The dotted lines represent the confidence intervals of the predicted values, whereas the solid line represents the predicted log odds of case status as a function of log DMP concentration.

Table 1

Semen parameter and demographic characteristics of controls and cases, Anhui, China, Winter 2004

	controls^a 95	cases^b 94	p
	Mean +sd	Mean +sd	
Percent total motile sperm	79.2 +5.8	52.6 +15.2	<0.0001
Sperm concentration (10 ⁶ sperm/ml)	78.6+69.5	17.2+11.52	<0.0001
Age	26.1 +2.7	26.0 +2.6	0.869
Abstinence (days)	2.9 +2.3	3.0 +2.4	0.685
Education	n(%)	n(%)	
Primary or less	10(11)	6(6)	0.589
Junior Middle	70(74)	73(78)	
Senior Middle or more	15(50)	15(16)	
Smoking			0.864
None	40(42)	37(39)	
Less than 10 cigs/day	26(27)	31(33)	
Between 10 & 20 cigs/day	15(16)	14(15)	
Alcohol use	30(32)	29(31)	0.914
Any work chemical exposure	41(43)	37(39)	
Passive smoke exposure			0.213
Home	60(65)	50(56)	
Other location	79(86)	76(85)	
Semen analysis delay ≥ 30 mins	76(80)	70(74)	0.364

^aControls were defined as individuals with both sperm concentration and total motility above the population medians after adjustment.^bCases were defined as individuals with both sperm concentration and total motility below the population medians after adjustment.

Table 2

Urine concentrations ($\mu\text{g/L}$) of OP metabolite concentrations among controls (n=95) and cases (n=94), Anhui China, Winter 2004.

	LOD	N > LOD	% Positiv.	Mean(SD)	GM(GSD)	Median	25 th perc	75 th perc	90 th Perc	Range
Diethylthiophosphate (DEDTP)	0.125									
control		14	14.7	0.15(0.10)	0.14(1.38)	<LOD	<LOD	<LOD	0.18	0.125-0.91
case		6	6.38	0.14(0.057)	0.13(1.25)	<LOD	<LOD	<LOD	<LOD	0.125-0.54
Dimethylphosphate (DMP)	0.25									
control		68	71.58	2.93(3.54)	1.39(3.80)	1.82	0.25	4.03	7.92	0.25-18.06
case		78	82.98	3.96(4.84)	2.02(1.46)	2.42	1.12	4.63	8.78	0.25-28
Dimethylthiophosphate (DMTP)	0.25									
control		89	93.68	4.14(7.33)	1.78(3.59)	1.84	0.77	3.97	7.89	0.25-50.83
case		88	93.62	3.36(5.69)	1.59(3.32)	1.41	0.76	3.97	6.54	0.25-41.66
Diethylphosphate (DEP)	0.125									
control		68	71.58	8.39(14.56)	1.98(7.42)	3.2	<LOD	8.15	23.8	0.125-81.24
case		67	71.28	6.90(9.19)	1.77(7.44)	2.69	<LOD	12.58	19	0.125-49.28
Diethylthiophosphate (DETP)	0.125									
control		93	97.89	23(29.99)	10.45(4.14)	10.61	4.35	28.82	59.2	0.125-171.64
case		89	94.68	18.7(21.8)	7.87(4.87)	9.63	2.82	26.08	51.3	0.125-87.5
Dimethylthiophosphate (DMDTP)	0.125									
control		19	20.00	0.23(0.26)	0.17(1.97)	<LOD	<LOD	<LOD	0.7	0.125-1.36
case		15	15.96	0.24(0.33)	0.17(2.03)	<LOD	<LOD	<LOD	0.62	0.125-2.27

^a controls were defined as individuals with both sperm concentration and total motility above the population medians after adjustment.

^b cases were defined as individuals with both sperm concentration and total motility below the population medians after adjustment.

LOD = limit of detection

All minimum values were below the LOD

Table 3

Relative odds of case status by 1 µg/L increase in 6 organophosphorous metabolites^a (95 controls^b and 94 cases^c)

	Crude		Adjusted ^d	
	OR	CI	OR	CI
Diethyldithiophosphate DEDTP	0.49	(0.16-1.51)	0.47	(0.14-1.54)
Dimethylphosphate DMP	1.24	(1.00-1.55)	1.30	(1.02-1.65)
Dimethyldithiophosphate DMTP	0.93	(0.74-1.17)	0.92	(0.73-1.18)
Diethylphosphate DEP	0.97	(0.84-1.12)	0.98	(0.85-1.14)
Diethylthiophosphate DETP	0.88	(0.73-1.07)	0.89	(0.72-1.09)
Dimethyldithiophosphate DMDTP	0.99	(0.65-1.49)	0.95	(0.62-1.47)

^a All the metabolites were log transformed

^b Controls were defined as individuals with both sperm concentration and total motility above the population medians after adjustment.

^c Cases were defined as individuals with both sperm concentration and total motility below the population medians after adjustment.

^d Adjusted for age, alcohol drinking, passive smoking, education, occupational chemical exposures, active smoking, abstinence from sexual activity, and time delay from ejaculation to laboratory analysis of semen.